

## 780 nm Low Power Diode Laser Irradiation Stimulates Proliferation of Keratinocyte Cultures: Involvement of Reactive Oxygen Species

Nili Grossman, PhD,<sup>1,2\*</sup> Naomi Schneid, B Med Sci,<sup>1,2</sup> H. Reuveni, MD,<sup>1</sup>  
S. Halevy, MD,<sup>1,3</sup> and R. Lubart, PhD<sup>4</sup>

<sup>1</sup>Skin Bank and Investigative Dermatology Laboratory, Soroka Medical Center and Faculty of Health Science, Ben-Gurion University of the Negev, Beer Sheva, Israel 84101

<sup>2</sup>Department of Microbiology and Immunology, Faculty of Health Science, Ben-Gurion University of the Negev, Beer Sheva, Israel 84101

<sup>3</sup>Department of Dermatology, Soroka Medical Center and Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel

<sup>4</sup>Department of Physics, Bar Ilan University, Ramat Gan, Israel 52900

**Background and Objective:** The purpose of this study was to determine irradiation parameters of a 780 nm low power CW diode laser (6.5 mW) leading to enhanced proliferation of cultured normal human keratinocytes (NHK). The possible role of reactive oxygen species (ROS) in this response was evaluated. **Study Design/Materials and Methods:** NHK were exposed to a single dose of 0 to 3.6 J/cm<sup>2</sup> (0–180 sec) of irradiation. Proliferation parameters studied were: incorporation of <sup>3</sup>H-thymidine during 6–24 hr following irradiation; percentage of dividing cells and number of cells, 24 hr and 48 hr following irradiation, respectively.

**Results:** Proliferation of NHK exposed to 0.45–0.95 J/cm<sup>2</sup> was significantly enhanced by 1.3–1.9-folds relative to sham-irradiated controls, as inferred from parameters studied. Exposure to other energy densities was considerably less effective in enhancing proliferation parameters. Added enzymatic antioxidants, superoxide dismutase or catalase, scavenging superoxide anions and H<sub>2</sub>O<sub>2</sub>, suppressed this enhanced proliferation. Added scavengers ( $\alpha$ -tocopherol acetate, scavenging lipid peroxidation, or sodium azide, histidine, mannitol, scavenging singlet oxygen, superoxide anions, and hydroxyl radicals, respectively), or N-acetyl cysteine, the thiol-reducing agent, suppressed the response, but to different extents.

**Conclusions:** The results indicate that 780 nm low power diode laser irradiation enhanced keratinocytes proliferation in vitro, with an apparent involvement of ROS in this response, and comparably, might be used to promote their proliferation in vivo to enhance wound healing. *Lasers Surg. Med.* 22:212–218, 1998.

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**Key words:** antioxidants; keratinocytes; low energy lasers; proliferation; reactive oxygen species

Contract grant sponsor: German-Israeli Foundation for scientific research and development (GIF); Contract grant sponsor: Ministry of Defense.

\*Correspondence to: Nili Grossman, Skin Bank and Investigative Dermatology Laboratory, Soroka Medical Center, P.O. Box 151, Beer Sheva, Israel 84101. E-mail: grossman@bgumail.bgu.ac.il

Accepted 2 February 1998

## INTRODUCTION

Low power lasers have been used as treatment for wound healing for the last 30 years [1]; however, there is still controversy regarding the efficacy and the specific role of these lasers in wound healing, [for review, 2–8]. Low power lasers emitting 780 nm, are among the least studied lasers, although several lines of evidence imply that this wavelength might be a potent tool to promote wound healing: In vivo, Ga-Al-As 780 nm laser enhanced granulation tissue formation and to some extent epithelization of chronic venous ulcers [9]. Recently, a 780 nm diode laser (Lasotronics, 30 mW) enhanced in vivo the initial rate of healing of cutaneous fissures due to various etiologies, relative to nonirradiated controls [10]. In vitro, a single exposure to 780 nm diode lasers promoted proliferation of cultured human [10] or murine fibroblasts [11], presumably echoing the in vivo activation of fibroblasts during wound healing [12]. The goal of the present study is to examine and demonstrate a stimulatory effect of a low power 780 nm CW diode laser (6.5 mW) on in vitro proliferation of epidermal keratinocytes, which participate in vivo in re-epithelization. As 780 nm irradiation may be absorbed by endogeneous acceptors, e.g., mitochondrial cytochrome oxidase [13], and thus may activate cellular functions via oxidative processes, we have investigated the presumed involvement of irradiation-induced reactive oxygen species (ROS) in the interaction of this low power diode laser and cultured keratinocytes [14–16].

## MATERIALS AND METHODS

### Tissue Culture Media

Tissue culture media, fetal calf serum (FCS), trypsin, and antibiotics were purchased from Biological Industries (Beth Haemek, Israel). Growth factors and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Superoxide dismutase (SOD) and catalase (Cat) were from Boehringer Mannheim (Mannheim, Germany).

### Cells Cultures and Growth Conditions

Normal human keratinocytes (NHK) were isolated from young foreskins (0–4 yr old) as described by Rheinwald and Green [17]. Growth medium was prepared as described [18]. Cells were incubated at 37°C, 8% CO<sub>2</sub>, and 95% humidity. Cells originating from ten different normal donors, at their second to fifth passage, were used

throughout this study. Each experiment was repeated at least three times for each cell line. Experiments were performed in 96-well clusters (Costar, Cambridge, MA), seeded with 13,000 NHK cells per well. This density allowed progression of the experiments while cells were in their logarithmic proliferative phase and did not reach confluence or switched to terminal differentiation.

### Irradiation Source and Irradiation Conditions

Irradiation source used was a 780 nm CW diode laser, constructed at the Department of Physics, Bar-Ilan University (Israel), emitting 6.5 mW, with a focal spot of 0.32 cm<sup>2</sup>, when positioned 2 cm above monolayers of cultured cells, seeded in wells. This spot size was identical to the surface area of a well in a 96-well cluster. Consequently, during experiments, one well was irradiated at a time, whereas all other wells stayed protected from direct light, covered by a black tape. To protect the cells from radiation scattering, each seeded well was surrounded with eight neighboring wells, containing trypan blue solution (0.4%). Diode laser power was monitored using a powermeter by Ophir (USA-Israel), with the probe positioned 2 cm below the diode tip, at the distance level of the cells from irradiation source.

Proliferating cells were irradiated 48 hr after seeding while in phosphate-buffered saline (PBS) for varying periods (0–180 sec), corresponding to 0 to 3.6 J/cm<sup>2</sup>. Following exposure, cells were refed with fresh growth medium and were further incubated. Sham-irradiated cells in PBS served as untreated controls. Experimental conditions were repeated in triplicates. In a few experiments, cells were exposed repeatedly to 0.35 J/cm<sup>2</sup> (a suboptimal dose) or to 0.6 J/cm<sup>2</sup> (an optimal dose), every 24 hr (total of three consecutive exposures), or every 48 hr (total of two consecutive exposures), with the first exposure occurring 48 hr following seeding, as described. Four days after the first irradiation, the number of cells was counted and compared to sham-irradiated controls.

### Proliferation Parameters

Total cell number per well was determined 24 hr or 48 hr following irradiation, after removal of the cells from the wells by trypsinization (0.25% trypsin–0.05% EDTA suspension) in a constant volume of 0.1 ml. Cells were counted microscopically using a counting chamber (Fuchs-Rosenthal, Germany). Three separate counts were performed for each well.

The fraction of cells at division was deter-

mined 24 hr following irradiation as the number of unseparated daughter cells divided by the total number of cells in each well. However, this latter parameter was associated with a large coefficient of variation, expressed as the ratio of the standard deviation (SD) to the mean of a triplicate, and ranged from 20% to 35% (e.g., in Fig. 1). Thus this parameter was studied only in five of the cell lines.

DNA synthesis following exposure to 780 nm irradiation was evaluated by assaying incorporation of  $^3\text{H}$ -thymidine (TdR; Rotem Industries, Israel) into trichloroacetic acid (TCA) precipitable material as described [19]. In short, 6 hr following irradiation,  $^3\text{H}$ -TdR (1  $\mu\text{Ci}/\text{well}$ ; 49.2 Ci/mmol) was added. At the end of 24 hr, cells were washed three times with cold PBS, and macromolecules were precipitated with 10% cold TCA. The amount of incorporated radioactivity was determined following resuspension of pellets in 0.1 N NaOH-0.1% SDS.

### Effect of Antioxidants

The effect of the following antioxidants was studied. Superoxide dismutase (SOD) and Catalase (Cat) were added at 50  $\mu\text{g}/\text{ml}$  following irradiation. Vitamin E ( $\alpha$ -tocopherol acetate) was added at 5  $\mu\text{g}/\text{ml}$  to the growth medium 48 hr before irradiation. Sodium azide (10 mM), histidine (10 mM), or mannitol (10 mM) were in PBS (30 min preincubation before irradiation) [20,21], and N-acetyl L-cysteine (NAC, 4 mM) was in PBS (120 min preincubation before irradiation [22]). In these experiments, two parallel sets of wells were seeded in triplicates and were exposed to various energy densities, ranging from 0–3.6 J/cm<sup>2</sup>, with one set treated with an antioxidant. When required, parallel triplicate wells with appropriate vehicle controls (0.1% ethanol for vitamin E treatment) were set for each experimental irradiation condition studied. Proliferation parameters, assayed as described, were incorporation of  $^3\text{H}$ -TdR 6–24 following irradiation, and total cell number 48 hr following irradiation.

### Statistical Analysis

For analysis, sample means and standard deviations (SD) were calculated for each experimental condition, repeated in triplicates. Results of repeated experiments in each cell line, were summarized in percentages, relative to their appropriate controls. Student's two-sided t-tests

were then used to test the differences among treatments and their controls.

## RESULTS

### Low Power 780 nm Diode Laser Irradiation Enhanced Proliferation of Cultured NHK

A single exposure of cultured NHK to 780 nm laser irradiation promoted their proliferation in an energy density-dependent response, relative to their sham-irradiated controls, as illustrated in Figure 1. In this experiment, the percentage of cells at division, determined 24 hr following irradiation (Fig. 1, upper panel), increased by 1.82–1.4-fold in wells exposed to 0.42–0.70 J/cm<sup>2</sup>, respectively, with a maximal and significant effect obtained following 0.42 J/cm<sup>2</sup> ( $P < 0.05$ ). Exposure to energy densities lower than this range did not affect the percentage of dividing cells, whereas exposure to  $>1$  J/cm<sup>2</sup>, significantly lowered it, e.g., by 50%, following 1.38 J/cm<sup>2</sup>. This specific decrease might be attributed to an increase, albeit insignificant, by  $1.15 \pm 0.08$  in the number of cells ( $P > 0.05$ ), 24 hr following irradiation. The total number of cells, determined 48 hr following irradiation (Fig. 1, lower panel), increased by 2.2–1.6-fold in wells exposed to 0.55–0.8 J/cm<sup>2</sup>, respectively, with a maximal and significant effect obtained following 0.55 J/cm<sup>2</sup> ( $P < 0.001$ ). Exposure to energy densities out of this range had a smaller and a less significant effect on this parameter (a 1.3–1.4-fold increase in this experiment;  $P < 0.05$ ) and was not preceded by a conspicuous increase in the percentage of dividing cells, 24 hr earlier. It is presumed that this was due to a combination of the relatively smaller increase expected in this parameter and the large coefficient of variation accompanying it.

In accordance with this proliferative response, a parallel modulation in the incorporation of  $^3\text{H}$ -TdR into TCA precipitable material during 6–24 hr following irradiation was observed, signifying DNA synthesis during the first cell cycle following exposure. In the four experiments summarized in Figure 2, the most effective energy densities in enhancing this parameter ranged from 0.6 to 1.2 J/cm<sup>2</sup> ( $P < 0.01$ ), with a maximal increase of  $1.58 \pm 0.08$  following 0.6 J/cm<sup>2</sup>.

The maximal and significant enhancement of all studied parameters was observed mainly following exposure to energy densities ranging from 0.45–0.95 J/cm<sup>2</sup>, irrespective of the NHK do-

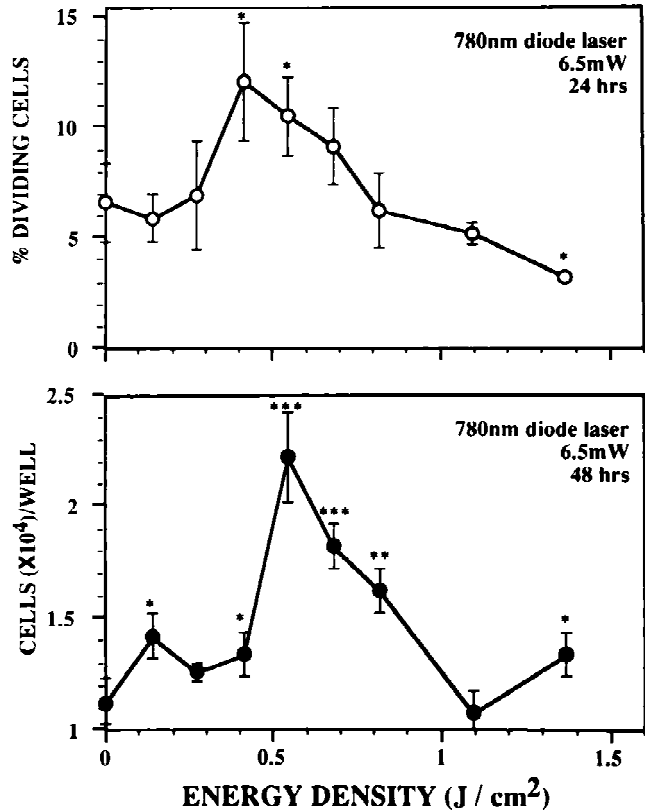


Fig. 1. Effect of 780 nm irradiation on proliferation of cultured NHK 48 hr following exposure. NHK were seeded in 96-wells clusters and 48 hr later were exposed to 780 nm diode laser irradiation while in PBS, as described in Materials and Methods. Cells were then replenished with fresh growth medium and were further incubated. The fraction of dividing cells (top) was determined 24 hr later under the microscope after removing cells from well by trypsinization, as described in Materials and Methods. At 48 hr following irradiation, cell number was determined (bottom). Significance of differences between irradiated and sham-irradiated controls: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

nor or their passages. The extent of increases in relative incorporation of  $^3\text{H}$ -TdR during the first 6–24 hr, or in cell number 48 hr following exposure, varied among NHK donors and ranged from 1.3–1.9-fold, presumably related to the natural variation of doubling times among normal primary cell lines. However, these variations, did not affect significantly the energy density-dependent response to low energy 780 nm diode irradiation. Taken together, these results suggested that within the first 48 hr following specific doses of 780 nm laser irradiation, the rate of DNA replication increased, resulting in a higher mitotic activity and leading to an increased number of cells.

This response was unique to proliferating

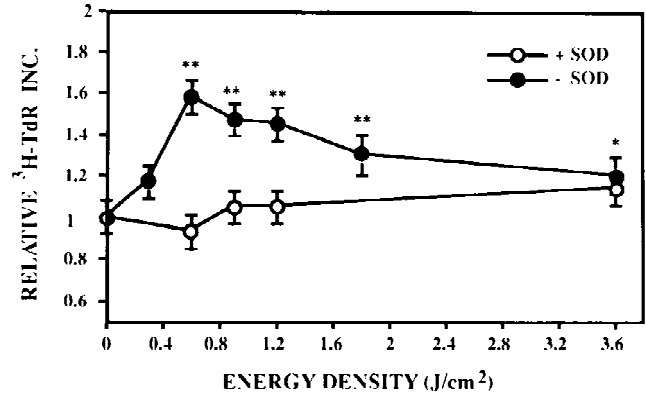


Fig. 2. Effect of SOD on 780 nm-enhanced DNA synthesis. NHK were seeded in 96-well clusters and were exposed to 780 nm diode laser while in PBS as described for Figure 1. SOD (50  $\mu\text{g}/\text{ml}$ ) was added to half of the wells following irradiation. Incorporation of  $^3\text{H}$ -TdR into TCA precipitable material during 6–24 hr following exposure was determined as described in Materials and Methods. Depicted are the average relative increments of  $^3\text{H}$ -TdR incorporation following of irradiation, relative to their nonirradiated control, with (open circles) or without (close circles) added SOD, in four experiments. Significance of differences between irradiated and their sham-irradiated controls was marked as in Figure 1.

cells and occurred independently of the presence of keratinocyte growth factors such as cholera toxin or EGF [17] in the experimental medium (not shown). Thus cells brought to quiescence by 72 hr incubation in medium supplemented with 0.5% FCS, or cells supplemented with 1% FCS, did not respond to 780 nm diode irradiation by enhanced proliferation, in any parameter studied (results not shown).

Repeated exposures to this 780 nm diode did not bring about a further increase in cell number beyond that observed following a single exposure to an optimal energy density (0.6  $\text{J}/\text{cm}^2$ ) of 780 nm (a  $1.44 \pm 0.07$ -fold increase determined 96 hr following the first exposure;  $P < 0.001$ ). An identical enhancement was observed in cells exposed once to 0.6  $\text{J}/\text{cm}^2$  and 24 or 48 hr later to suboptimal energy density (0.35  $\text{J}/\text{cm}^2$ ) ( $1.48 \pm 0.12$ ;  $P < 0.001$ ). Cells exposed once to this suboptimal level were enhanced only by  $1.26 \pm 0.05$  ( $P < 0.01$ ) relative to their controls. In fact, proliferation of NHK subjected to repeated exposures to 0.6  $\text{J}/\text{cm}^2$ , either twice or three times within 48 hr, was not enhanced relative to their sham-irradiated control ( $0.92 \pm 0.11$ ). These experiments suggested that NHK were refractive to any putative additional enhancement of growth during the first 3–4 days following the first exposure to 780 nm irra-



**TABLE 1. Antioxidants and Suppression of 780 nm Diode Laser-Enhanced Proliferation of Cultured NHK\***

Effector	Relative enhancement in:		Relative suppression	Significance
	Control cultures	Treated cultures		
SOD (50 $\mu\text{g/ml}$ )	$1.58 \pm 0.15$	$1.05 \pm 0.14$	91%	$P < 0.01$
Cat (50 $\mu\text{g/ml}$ )	$1.60 \pm 0.15$	$1.00 \pm 0.10$	100%	$P < 0.01$
Vitamin E (5 $\mu\text{g/ml}$ )	$1.32 \pm 0.15$	$1.07 \pm 0.11$	78%	$P < 0.05$
Azide (10 mM)	$1.31 \pm 0.07$	$1.08 \pm 0.08$	78%	$P < 0.01$
Histidine (10 mM)	$1.93 \pm 0.20$	$1.34 \pm 0.10$	64%	$P < 0.01$
Mannitol (10 mM)	$2.00 \pm 0.20$	$1.08 \pm 0.10$	92%	$P < 0.01$
NAC (4 mM)	$1.49 \pm 0.05$	$1.07 \pm 0.06$	86%	$P < 0.01$

\*NHK were exposed to 780 nm diode laser while in PBS for various periods, 48 hr following seeding in tissue culture plates. Treatments with antioxidants were performed following irradiation (SOD, Cat); 48 hr (vitamin E), 120 min (NAC), or 30 min prior to irradiation (all the others). At the end of exposure, growth medium was added back. Incorporation of  $^3\text{H}$ -TdR during the first 6–24 hr following exposure was determined. The data presented are relative increments of  $^3\text{H}$ -TdR incorporation following exposure to  $0.6 \text{ J/cm}^2$ , which gave the highest enhancement in untreated controls. All experimental points were repeated in triplicates, and the results are averages  $\pm$  SD of four experiments each.

diation. Moreover, repeated exposures to higher energy densities, might interfere with expression of the proliferative response to a single exposure and suppressed it.

#### Antioxidants Suppressed the Stimulatory Effect of 780 nm Low Power Laser Irradiation

In order to examine the potential involvement of ROS in the proliferative response to 780 nm, we have determined the effect of various antioxidants on enhanced  $^3\text{H}$ -TdR incorporation following irradiation. In a typical experiment (as visualized in Fig. 2), the addition of exogenous SOD to scavenge putatively produced superoxide anions [23] following exposure to 780 nm irradiation, significantly suppressed the enhanced radiolabel incorporation. Similarly, exogenous SOD suppressed the increase in cell number 48 hr following exposure to 780 nm diode laser: a 45-sec irradiation ( $0.9 \text{ J/cm}^2$ ) resulted in a  $1.60 \pm 0.05$  increase in the number of cells 48 hr later, whereas in the presence of SOD, the increase was only by  $1.12 \pm 0.06$  ( $P < 0.001$  between the two responses).

Table 1 summarizes the results of similar experiments, depicting the maximal effect of 780 nm irradiation on subsequent  $^3\text{H}$ -TdR incorporation with and without the following antioxidants: SOD; catalase (Cat) that eliminates  $\text{H}_2\text{O}_2$  [22]; Vitamin E ( $\alpha$ -tocopherol acetate) that scavenge cell membrane lipid-derived radical intermediates [23]; sodium azide, known to scavenge singlet oxygen, and almost as effectively, hydroxyl radicals [21], and which was shown to prevent iron-dependent formation of lipid peroxidation [20]; histidine, scavenging singlet oxygen (rate constant  $3.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ) and more specifically hy-

droxyl radicals (rate constant  $5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ) [20]; Mannitol, known to scavenge hydroxyl radicals [21]; and N-acetyl L-cysteine (NAC), a thiol-reducing agent [22], shown to protect tissue against photodynamic damage caused by porphyrin-based photosensitizers [24]. All the antioxidants examined in these experiments suppressed the 780 nm-enhanced TdR incorporation, as well as the increase in cell number (not shown). No suppression was observed following treatment with DMSO (4%) (not shown), another potent hydroxyl radical scavenger, presumably due to its poor excess to target molecules in the cell [21].

#### DISCUSSION

The initial aim of this study was to determine irradiation parameters leading to enhanced proliferation of cultured NHK following 780 nm low power diode laser irradiation and to examine the presumed involvement of ROS in this proliferative response. This in vitro enhanced proliferation may reflect the proliferation phase of basal keratinocytes, an important component in vivo of reepithelization [12]. The low power CW 780 nm diode laser (6.5 mW, with an effective focal spot of  $0.32 \text{ cm}^2$ ) used in this study allows a precise determination of irradiation parameters that stimulate proliferation of skin-derived cells better than a previously studied diode (Lasotronics, emitting 25 mW at a focal spot of only  $20 \text{ mm}^2$ ) [10]. Three proliferation parameters measured within the first two doubling times following exposure were examined: incorporation of radiolabeled TdR into macromolecules, signifying DNA synthesis during the first doubling time, percentage of cells at division, and total number of cells 24 hr and 48 hr

following irradiation. A maximal and significant enhancement of all studied parameters was observed mainly following exposure of NHK to energy densities in the range of 0.45–0.95 J/cm<sup>2</sup>, relative to their sham-irradiated controls. The energy-density dependent response described herein suggested that a threshold energy density level of 780 nm irradiation was required for initial enhancement, whereas energy densities >1 J/cm<sup>2</sup> were less effective in stimulating proliferation, implying possible inhibitory effects at >4 J/cm<sup>2</sup>. In accordance, cells were refractive to any further stimulation by repeated exposures to irradiation.

This proliferative response to this 780 nm diode laser is not unique to young NHK, but rather a general response of several cell types, including normal human fibroblasts, adult human keratinocytes, rat epithelial cells, and calf endothelial cells (Grossman et al., unpublished observation). However, the extent of enhancement within each of these cell types seemed to be dependent and was inversely related to increased donor age, or at times, to passage examined, or to increased doubling time. Unlike studies with other near infrared lasers [25], this proliferative response could not be observed in quiescent or slowly proliferating NHK. In addition, parameters of affective irradiation differed among various cells types, presumably due to differences in their spectral characteristics.

The proliferative response to low energy 780 nm corresponds to biostimulatory effects that were demonstrated following irradiation by HeNe lasers and low energy visible and near infrared lasers and diode lasers [e.g., 6,11,14,15, and reviewed in 2–8]. At the same time, others reported of failures to stimulate proliferation or any other biological responses [reviewed in 2–8], e.g., a recent report on the lack of effect of a 830 nm Ga-Al-As laser on cultured fibroblasts and keratinocytes [26]. Some of these contradicting reports may be explained by the variety of irradiation sources and irradiation modalities examined, detection methods used, and their sensitivity [27].

In a search for the intracellular mechanisms involved in this biostimulatory effect, we examined the hypothesis that ROS induced by exposure to 780 nm irradiation were involved, specifically, of an energy density-dependent ROS production. It was assumed that suppression of the proliferative response by added extracellular antioxidants might indicate the involvement of those specifically scavenged ROS, or their downstream intermediates. This experimental ap-

proach is among the few applicable in a model system with a limited irradiation area and low number of cells. By a similar approach, the involvement of ROS in induction of responses to irradiation has been elucidated (e.g., on induction of heme oxygenase in human fibroblasts following UVA [20,21], or on decreased <sup>3</sup>H-TdR incorporation in the macrophage-like cell line U937 following 660 nm laser irradiation [16].

The results presented in Figure 2 and Table 1 suggest that a variety of ROS intermediates may be involved in the stimulatory effect of 780 nm laser irradiation on NHK proliferation. The scavenging by various antioxidants might suggest a general nonspecific effect. Alternatively, the finding that antioxidants scavenging singlet oxygen and superoxide anions suppressed the 780 nm induced proliferation might imply that a cascade of ROS production, beginning with these intermediates was activated by 780 nm, similar to the effect of visible and near-UV light [13–16,20–21]. The varying levels of suppression exerted by these antioxidants (Table 1) might indicate the relative importance of various ROS in this presumed cascade of events, as well as their differential susceptibility to scavenging.

The downstream signal transduction pathways participating in this near-infrared diode laser has not been resolved yet. But the results suggest that at least two key transcription factors, AP-1 and NFκB, shown to be activated following oxidative stress, UV irradiation, and photodynamic therapy [22,24,28,29] might be involved. Activation of these factors was shown to be suppressed by α-tocopherol acetate and NAC (AP-1 and NFκB, respectively; 22), two antioxidants that suppressed the 780 nm enhanced proliferation.

In conclusion, this study provides a conclusive evidence for stimulated proliferation of cultured skin cells following their exposure to specific doses of low power 780 nm irradiation and for the involvement of ROS in this proliferative response. It may thus contribute a clue for the beneficial effect of low power 780 nm diodes recently included in various commercial low power laser systems, clinically used for enhancement of wound healing.

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